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ORIGINAL ARTICLE

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During ischemia-reperfusion in rat kidneys, heat shock response is not regulated by expressional changes of heat shock factor 1

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Abstract Ischemia-reperfusion injury is known to induce the inducible form of the 70 kDa heat shock protein HSP70i (or HSP72) mainly via rapid activation of heat shock transcription factor 1 (HSF1). However, little is known about the regulation of the HSF1 gene. We therefore studied the time course of HSF1 mRNA transcription and its relation to the expression pattern of the HSP70i mRNA in the renal cortex, this being the most vulnerable and functionally most important part of the kidney, after different periods of unilateral renal ischemia (10–180 min) and reperfusion (up to 60 min) in male Wistar rats (10 weeks old). Immediately after ischemia there was a significant induction of HSP70i genes. While HSP70i expression constantly increased (up to 4-fold) during reperfusion, even to a higher extent with prolongation of ischemia, HSF1 mRNA remained constitutively expressed under all conditions. Thus, we conclude that during ischemia-reperfusion in rat kidneys, the heat shock response is regulated by other means than expressional changes of HSF1.

Key words Ischemia-reperfusion · Heat shock · HSP70 · Heat shock factor (HSF) · Kidney

Introduction

The temporary discontinuation of renal blood supply is a consequence of several clinical conditions such as kidney transplantation, aortic aneurysm surgery, or hypotension due to resuscitation. The organ response to injury is modulated by the expression of genes that are necessary for cell- and organ survival during and after the insult. In this context, expression of heat shock proteins (HSP) -a universal stress response remarkably conserved during the entire evolution – is extremely important and probably the best characterized system of cellular defense. HSP belong to a diverse family of inducible and constitutive stress proteins [20, 24, 26]. In mammalian cells, the inducible 70-kDa HSP (HSP72 or HSP70i) is the most abundant HSP [48] and is the HSP most closely linked to cytoprotection [38] from a variety of dangerous events like thermal injury or ischemia-reperfusion. HSP72 expression is mediated mainly via activation of heat shock factor 1 (HSF1). HSFs are coded by a small family of genes with up to 5 members. Two of them were identified in mammalian cells and cloned in 1991 [36, 43]. Since then, many experiments have been conducted to investigate the activation mechanisms of HSFs such as phosphorylation, translocation, oligomerisation, and binding to heat shock elements [21, 25, 30, 45, 50]. In mammals, HSF1 is considered responsible for stress-induced gene activation during ischemia-reperfusion [34]. However, little is known about HSF1's gene expression [9]. Although heat shock (HS) response has been extensively studied in the heart, liver, brain, lung, and other organs and tissues, information about HS-gene regulation in kidney is limited. We therefore studied the time course of HSF1 mRNA and HSP70i mRNA expression in renal cortex, this being the most vulnerable and functionally most important part of the kidney, after different periods of renal ischemia and reperfusion in comparison with sham-operated rats.

Materials and methods

In a former experimental study of our group, the expressional regulation of the two coding genes for HSP70i – HSP70–1 gene and HSP70–2 gene were investigated by analyzing the time course of HSP70–1 and –2 mRNA expression and its relation to cellular ATP levels [2]. Renal cortical tissues chosen at random from these experiments (n = 4–7 for each reperfusion group) were now investigated for the time course of HSF1 mRNA transcription and in these specimen HSP70i expression was quantified, too.

Animal preparation and experimental set-up

Unilateral warm renal ischemia was conducted on male Wistar rats (10 weeks old) for 10, 40, 60, 120 and 180 min under pentobarbital anesthesia (100 mg/kg bw i.p.). After reperfusion times of 0, 5, 10, 30 and 60 min (n = 10 for each reperfusion group), left nephrectomy was performed. All surgical procedures were performed from 9.00–11.00 p.m. and body temperature was kept constant at 37 °C throughout. Kidneys from rats not exposed to ischemia-reperfusion but to anesthesia served as controls. During the animal experiments, principles of laboratory animal care were followed, and the current version of the "German Law on the Protection of Animals" was applied.

RNA-Isolation

All experimental steps were performed under RNase-free measures. Total RNA was isolated from renal tissue by mechanical crushing in liquid nitrogen, homogenization in guanidinium-isothiocyanate solution and centrifugation through a cesium-chloride cushion [4]. Concentration of RNA was calculated from A_{260} values. Quality of isolated RNA was assessed by agarose gel electrophoresis.

Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA (each RT-reaction containing 250 ng) was reverse transcribed in a thermocycler (Trioblock, Biometra, Göttingen, Germany) using SuperScript Plus II RNAse H$^-$ Reverse Transcriptase (GIBCO-BRL, Eggenstein, Germany). 1 mm of each dNTP (Pharmacia, Freiburg, Germany), 2.5 U Rnasin (GIBCO-BRL, Eggenstein, Germany), 0.5 mg of random hexamer oligonucleotide primers and 15 U of reverse transcriptase were added to "1 × reverse transcription buffer" in a total final volume of 20 µl. The reaction was incubated at 37 °C for 10 min, then for 20 min at 42 °C, stopped by heating at 95 °C for 10 min and finally chilled on ice. This first strand cDNA reaction was used as a template for PCR amplification containing the following components: 1 × PCR-buffer (complete); 12 µM each dNTP; 5 pmol each specific primer (GIBCO-BRL, Eggenstein, Germany); 2 U Taq-DNA-Polymerase (Pharmacia, Freiburg, Germany). In a thermocycler (PE 9600, Perkin Elmer), amplification cycles were performed after an initial denaturation of 2 min at 95 °C, 30 s denaturation at 94 °C, 30 s primer annealing, 30 s extension at 72 °C. Reactions with equal amounts of not reverse transcribed RNA were included to control for amplification of contaminating genomic DNA. This is necessary because HSP70 genes are intronless. To determine the cycle numbers for PCR products remaining in a linear phase of amplification, the PCR products of each gene were sampled at 20, 25, 30, 35, or 40 cycles (95 °C for 1 min, annealing temperature for 1.5 min and 72 °C for 1.5 min). After amplification, 20 µl of the PCR products were separated by 1 % agarose gel electrophoresis and photographed.

Primers

HSF1 primers were designed and (GIBCO BRL-Life Technologies) according to their sequences; primers for HSP70 were chosen for amplification of HSP70–1 and HSP70–2 messages concomitantly [26, 2, 47, 49]. The primers for Gap-DH were used as previously [2]. Primer sequences and annealing temperatures (AT) were as follows:

HSF 1	Reverse	5'CTGCTGTAG-GCT GGA-GATGG 3'	AT: 60 °C
	Forward	5'TGAAGCAC-GAGAAC-GAGGC 3'	
HSP70–1&2	Reverse 5' CGCTGCGAGT-CGTTGAAG-TAG 3'	AT: 62 °C	
	Forward	5' GTCGGACAT-GAAGCACTGGC 3'	
Gap-DH Reverse	'5'TGACCTTGC-CCA-CAGCCTTG 3'	AT: 65 °C	
	Forward	5-'CATCAC-CATCTTCCAG-GAGCG 3'	

PCR fragment elution and DNA-sequencing

To verify the correctness of amplified fragments, we did not only check the expected size but also determined the nucleotide sequences of PCR-fragments after elution from the agarose gel (with dialysis membrane) using the Dye Terminator Cycle Sequencing Ready Reactions (Perkin Elmer, Langen, Germany) and ABI automated sequencer (Applied Biosystems, Weiterstadt, Germany).

Data

Semi-quantitative RT-PCR was evaluated by scanning of polaroid negatives of the gel images using a laser densitometer and a computer based imaging system (Molecular Dynamics, Krefeld, Germany). All mRNA levels (relative units normalized to Gap-DH) are given as mean ± SEM. Statistical significance was determined using the unpaired Student's t-test.

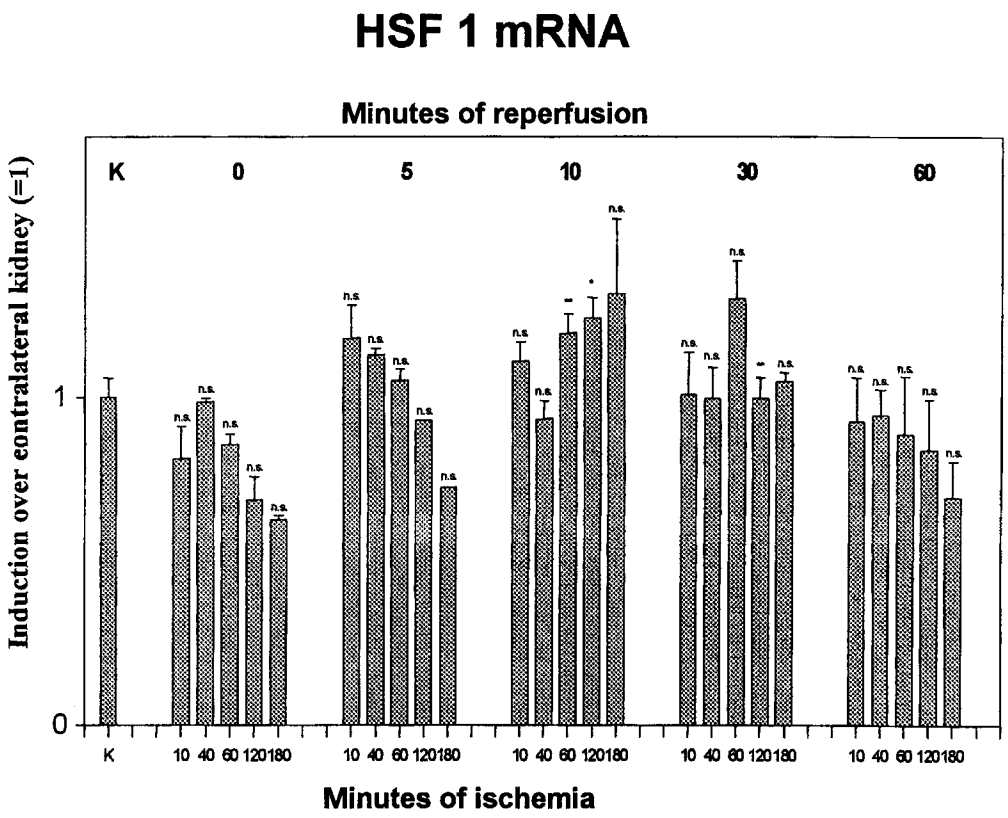

Fig. 1 Time course of HSF1 mRNA induction after ischemia and reperfusion in male wistar-rat-kidneys measured with semiquantitative RT-PCR as relative induction compared with the basic expression level (= 1) in non-stressed kidney tissue (* $P < 0.01$, ** $P < 0.05$, n.s. non-significant)

Results

HSF1 mRNA (summarized in Fig. 1)

Even though minor changes could be measured, we could not detect any trend for an altered regulation of HSF1 expression in our experiments. The HSF1- and mRNA values did not differ significantly from control values during the entire course of reperfusion and thereafter.

HSP70i mRNA (summarized in Fig. 2)

At the end of all ischemic periods (10, 40, 60, 120 and 180 min) and also after 5 min of reperfusion there was no significant change in the expression levels compared with controls. But then, regardless of duration of the ischemic period (except for 10 min of ischemia), HSP70i mRNA increased about 2-fold after 30 min of reperfusion. After 1 h of reperfusion there was an almost 3-fold higher expression. Kidneys exposed to 10 min of ischemia had a maximum expression after 30 min of reperfusion with a 1.5-fold increase that remained on the same level by 60 min of reperfusion. In kidneys exposed to 180 min of ischemia HSP70i mRNA expression did not exceed the values after 60 and 120 min. This augmentation of HSP70i mRNA (= HSP70-1&2 mRNA) expressions in the samples selected for the present evaluation confirmed our earlier data on HSP70 expression obtained in other renal samples from the same experiments [2].

Discussion

As discussed in our previous investigations [2], only young rats were investigated because the extent of stress-inducible HSP70 expression constantly decreases with age [28]. Since constitutive expression of HSP70 is usually at very low levels [13, 29], and some authors even report HSP70 to be undetectable in normal rat kidneys [15], or at least in the renal cortex [42], the RT-PCR was the method of choice. Homogenates of renal pole tissue (mostly cortex) were investigated, because after ischemic injury tubular/glomerular expression is reported [12], although interstitial HSP70 induction in rat kidneys is described too [6, 22]. Amounts of constitutively expressed HSP70 are lowest in the cortex compared with the inner medulla, where the highest values are found; after ischemia, even a decrease of HSP70 is observed in the inner medulla, and during reperfusion, original levels are barely reached [31]. Contralateral non-ischemic kidneys served as controls since there is

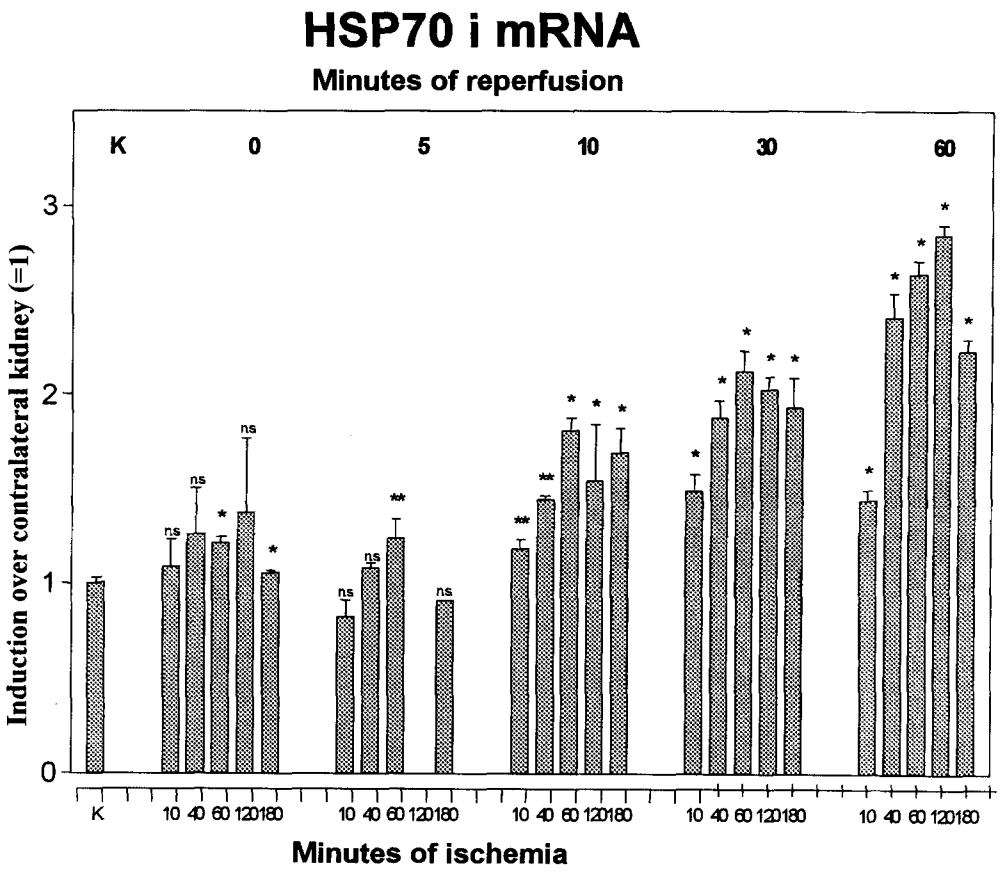

Fig. 2 Time course of HSP70i mRNA induction after unilateral warm ischemia and reperfusion in male wistar-rat-kidneys measured with semiquantitative RT-PCR as relative induction compared with the basic expression level (= 1) in non-stressed kidney tissue (* $P < 0.01$, ** $P < 0.05$, n.s. non significant)

multiple evidence that drugs can be modulators of the heat shock response and influence of anesthesia and laparotomy is not known. Temperature changes can result in a strong heat shock response [7], so that body temperature was carefully kept at 37 °C. Finally, although there is no circadian rhythm of HSP70 expression in eucaryotes [37], all experiments were performed from 9.00–11.00 p.m..

The expression of some HSFs are heat-inducible [4], i.e. in tomato, two of three described different HSF are inducible [11, 40, 41, 46]. Recently some authors supplied evidence, that mammalian HSF1 might be regulated on a transcriptional level too: for instance, overexpression of HSP70i in gene-transfected human epidermoid cells leads to downregulation of HSF1 [51] and heating of human epidermoid cells induced a significant increase in HSF1 mRNA [9]. However, we could not measure any significant changes in the level of HSF1 expression throughout our experiments. This correlates with the results of many other authors experimenting with other organs in vivo [5, 19, 21 32, 33]. These authors found HSF1 constitutively expressed under a variety of different stressing circumstances. A cell culture system may be equipped with different regulatory mechanisms compared to organs in situ. The kind of stress applied in our study – ischemia and subsequent reperfusion – may not be the adequate stimulus, resulting in HSF1 mRNA upregulation in vivo. One might even speculate that HSF1 expressional regulation alone under certain conditions could possibly be insufficient for eliciting the full stress response: Simulated upregulation (gentransfection with HSF1 leading to its overexpression in human epidermoid cells) did not result in increase of HSP70i mRNA [51].

At this point it is important to mention that the accuracy of quantification of mRNA by semiquantitativeRT-PCR is limited, even though it is by far one of the most sensitive methods, especially if only very small samples are available [17]. Thus, it is not possible to exclude minimal changes (as shown by us on a non-significant level) in HSF1 specific mRNA after ischemia-reperfusion. Another method could be to measure a tremendously high number of samples, but the logic of known biomechanisms does not justify such an approach since for HSFi transcriptional activity there are positive and negative regulatory mechanisms by posttranslational modifications such as in HSF1 phosphorylation [11, 14, 15, 18, 21, 23, 35] or dephosphorylation [19, 44], HSP-binding to the DNA bound HSF1 trimer [41], temperature changes [33] and multiple influences by cofactors

[5, 32]. Such modifications can act more rapidly and may be more effective in HSP induction than expressional changes of HSF1.

Our quantification of HSP70 mRNA illustrates that the expected heat shock response had occurred in the tissue specimens selected for HSF1 analysis. Heat shock response in rat kidney has been demonstrated by many authors [15, 27, 31, 42, 52], but no report of its activation via HSF upregulation after ischemia-reperfusion injury is available to our knowledge.

In accordance with other groups [3, 16, 42] that were able to demonstrate a heat shock response, we could clearly show induction of HSP70i mRNA expression in rat kidney after ischemia-reperfusion, which was dependent on the duration of ischemia in the range of 60–120 min, while after 180 min, irreversible ischemic damage to kidney led to a lower expression of HSP70i mRNA (Fig. 2) [2]. After 10 min of ischemia, the expression of HSP70i mRNA during subsequent reperfusion, was low and the peak expression was reached already after 30 min of reperfusion, while after longer periods of ischemia the peaks could not be observed during 60 min of reperfusion (Fig. 2) [2]. This weak and temporary heat shock response is in accordance with the fast ATP regeneration in rat kidneys after 10 min of warm ischemia [1]. Normalization of HSP72 values after ischemia-reperfusion was reported by Schober et al. after 7 days [42].

The missed induction immediately after ischemia confirms previous investigations; it was no surprise that there was no significant increase after 5 min of reperfusion, since it is known that during ischemia, and in the early phase of reperfusion, protein synthesis is downregulated. DeGracia et al. [8] showed that this is not due to an altered ribosomal function but that, on the contrary, ribosomes are fully functional, at least in vitro, during the whole reperfusion period. It was suggested that the stop of de novo synthesis is a protective mechanism to prevent misfolding of proteins and a possible chaperone release of ribosome-associated HSP could nevertheless support the cellular defense [39]. The HSP70i mRNA therefore probably remains constant during ischemia, when protein synthesis is depressed, and can be reinitiated by the newly synthesized mRNAs during reperfusion to protect the cells from reperfusion injury.

Thus, we conclude that during ischemia-reperfusion in rat kidneys there is a heat shock response which is dependent on the duration of ischemia and which is regulated in other ways than by expressional changes of HSF1.

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